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14. ABSTRACT

We have made strong progress on each of the proposed Aims. We have successfully generated reagents for, and begun the evaluation of, several of the regulatory nodes that comprise the mTOR pathway including Rheb, Pten, Raptor, and Rictor. We have found that Rheb up- regulation weakly phenocopies NF1 loss, while PTEN loss shares many similar characteristics. We have found conditions to induce EndMT in primary endothelial cells. While data to date do not support the hypothesis that this is being modulated by the loss of NF1, this analysis is ongoing. Preliminary results suggest that the loss of NF1 may alter the balance of ALK receptor signaling and that the loss of ALK5 signaling can phenocopy the morphogenic defects seen with NF1 loss. Lastly we have successfully established the genetic lines required for our in vivo experiments and we are poised to begin the Cre-induction in the first experimental cohort.

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Introduction:

NF1 plays an important role in regulating the vascular endothelium. NF1 is clinically associated with multiple vasculopathies including malformations, aneurysms, and hypertension. Consequently there is a markedly elevated risk of cerbrovascular accidents(Friedman et al., 2002). NF1 haploinsufficient mice show exaggerated angiogenic responses(Wu et al., 2006) and data have been published suggesting that shRNA mediated knockdown of NF1 can augment growth factor mediated Ras activation and downstream signaling in endothelial cells(Munchhof et al., 2006). We have recently published that activation of Ras in primary endothelial cells is sufficient to drive a pro-survival, pro-proliferative phenotype that disrupts normal vascular morphogenesis(Bajaj et al., 2010b). Our data also document that loss of NF1 in primary human endothelial cells results in autonomous proliferation and activation of multiple signaling networks. These changes result in abnormal vascular morphogenesis. These effects are driven by the specific activation of Ras and are reversed by expression of the GRD of NF1 and by low doses of the mTOR inhibitor rapamycin (Bajaj et al., 2012) Our central hypothesis is that mTOR activation is a crucial component of vascular abnormalities associated with NF1 and contributes to an altered vascular microenvironment that promotes much of the pathology associated with the disease. Moreover we hypothesize that a mechanistic consequence of the loss of NF1 is an alteration in the TGF-b signaling axis, that promotes abnormal vascular differentiation and may alter the tumor microenvironment. We address these central hypotheses in this project with three Specific Aims:

1. ***Investigate the molecular regulation of mTOR-related signaling in NF1-deficient human endothelial cells and its role in altered endothelial cell function.*** Using molecular modulation of componenets of the mTOR pathway as well as clinically available pharmaceuticals, we are determining the critical regulatory pathways that drive the changes seen following the loss of NF1 in endothelial cells.
2. ***Determine if NF1-deficient human endothelial cells have altered responses to TGF-b.*** We are investigating whether the loss of NF1 alters the response to the TGF-b signaling axis and determining if this alters the process of Endothelial-Mesechymal Transition (EndMT).

3. ***Evaluate the consequences of bi-allelic loss of NF1in the vascular endothelium.*** Using endothelial specific, tamoxifen inducible Cre-lox technology, we are determining the effects of losing a second allele of NF1 in the vascular endothelium of the adult mouse. This will be the first model of NF1 loss in the adult endothelium and can serve as a model system for investigation of both cardiovascular effects and the tumor microenvironment.

Body:

Aim 1.

As outlined above, a principal objective of the first aim is to better understand the relationship of mTOR signaling in endothelial cells and determine which aspects of this complicated regulatory network were being dysregulated. To that end a high priority of the first years' work, as outlined in the SOW, is to establish and characterize a number of these reagents we are using to manipulate this system, with respect to signaling and functional outcomes. We are well on our

way toward achieving these objectives. The individual pathways we are targeting and our progress are highlighted below

Rheb: We have generated a lentiviral vector that expresses an epitope (FLAG)-tagged WT Rheb construct under the control of a doxycycline inducible promoter. We have used this virus to infect and sort primary endothelial cells for expression of this construct. We have characterized the inducibility and signaling of this construct. As shown in **Figure 1A**, we get excellent induction of this construct following addition of doxycycline to the media. Our anticipated outcome was that upon expression of Rheb, we would induce a strong activation of mTOR as had previously been published (Sato et al., 2009; Sato et al., 2008). However, we found that the level of phosphorylation of S6, a downstream target of mTOR was quite modest under non-stimulated conditions. While there is clearly inducible activation of S6 phosphorylation, **Figure 1B**, it is less than that typically seen following manipulation of other mTOR related signals, including what we have published following the loss of NF1(Bajaj et al., 2012) and Ras (Bajaj et al., 2010a). *We hypothesize that this may indicate a high level of control over Rheb by the endogenous regulators TSC1/2. A potential augmentation of these studies would include an shRNA knockdown of TSC1/2 to determine if this results in a more robust activation of mTOR.* We have analyzed a number of related signal transduction pathways and the response to complete growth media containing serum, as shown in the Western blot in **Figure 1B**. A modest upregulation of eNOS phosphorylation is consistently noticed, however we have not seen any consistent upregulation of phosphoAKT, suggesting that at the levels our Rheb expression system is able to generate, only TORC1 and not the TORC2 axis seems to be activated. This is particularly interesting in light of the data shown in **Figure 2**. In these experiments, we performed the same type of doxycline induction as indicated in Figure 1 except we used a sub-maximal agonist, VEGF. Here it can be appreciated that the level of phosphoS6 activation is similar to that seen for VEGF. Interesting under these conditions we see a subtle but seemingly reproducible finding that phospho-AKT stimulated by VEGF, actually seems repressed following VEGF treatment. This would be unexpected. We are in the process of repeating this experiment a number of additional times in order to gather enough quantitative data to judge this effect accurately. We have also evaluated several of the functional aspects of endothelial cells following Rheb expression, as shown in **Figure 3**. We have seen negligible effect on cellular proliferation in complete media. Rheb expression is not sufficient by itself to drive cell proliferation and the augmentation of the growth media induced response is not significant. We are still in the process of evaluating the response to VEGF, a less potent proliferative agonist for potentiation. Interesting despite the modest effects on cell signaling and proliferation, we do find a consistent morphological defect in the cells expressing Rheb, as shown in **Figure 3B**. The cells consistently form some "island" or sheet like structures and have stubbier and thicker branching structures, suggesting that their branching morphogenesis is compromised following activation of mTORC1, largely in the absence of any detectable pERK and pAKT signaling, implying that TORC1 activation is a critical control point for morphogenesis. Our goal is to continue to refine our knowledge of this process by evaluating the effects of the pharmacologic compounds rapamycin, metformin, and lovastatin on this process, to determine if it can be normalized.

PTEN: Another goal of Aim 1is to determine the effects of knocking down PTEN, as this would be predicted to result in modest AKT activation, while not effecting the Erk signaling that is seen

following NF1. This again provides critical information on isolating the signals responsible downstream of NF1 for the abnormal morphogenesis. We have created a vectors for the expression of mir-based shRNA targeting PTEN. We have used these vectors to selectively infect and select primary human endothelial cells, and achieve a highly efficient knockdown of PTEN. This data is shown in **Figure 4A**. We have analyzed the effect on signals in response to both complete growth media, as well as to VEGF. The loss of PTEN is sufficient to induce an accumulation of phosphorylated phospho-S6 and Akt and phosphorylation of eNOS. In addition it significantly enhances these signals when the cells are stimulated with VEGF, though it until our quantitative analysis is complete it is unclear if this is additive or synergistic. We evaluated the effects on proliferation and found that the knockdown of PTEN was unable to induce a proliferative response on its own, however it significantly augmented the stimulated proliferative response, consistent with the augmentation of cell signaling **Figure 5A**. The sensitivity of this super stimulation is currently unknown but we suspect it will be sensitive to rapamycin, similar to NF1 knockdown. We also evaluated the effects of the loss of PTEN on the vascular morphogenic response of these cells and similar to the loss of PTEN, we find that the response is completely abrogated – we found little to know tube like structures upon knocking down PTEN, rather the cells remained as a planar, sheet-like monolayer – the cues for normal sprouting and tube formation are apparently disrupted **Figure 5B**. Like cells lacking NF1, this morphological defect is normalized by treatment with low-dose rapamycin, which directly inhibits mTORC1 **Figure 6**., but they appear to be largely unaffected by the indirect modulator of mTORC activity, metformin, which activates AMPK, which in turn can phosphorylate TSC1/2 and enhance mTORC suppression. The validation of the presumed pharmacologic effects and data replication quantification are on-going for these experiments.

Raptor: To provide a molecular means to interfere with mTORC1 complex, we developed an shRNA construct that would target Raptor. This was subcloned into a doxycycline-inducible lentiviral vector and primary cultures of HUVEC cells were infected and sorted by FACS. The efficiency of this construct is shown in **Figure 7**A. Control cells or those infected with the Raptor shRNA were cultured in the presence doxycycline under exponentially growing conditions when mTOR activation is maximal. As can be seen the shRNA nearly eliminates expression of Raptor and significantly curtails phosphoS6, a marker of mTORC1 activation, while slightly enhancing Akt phosphorylation at S473, a site for mTORC2 phosphorylation mediated by Rictor. This slight enhancement is presumably mediated by the increase in mTOR available to complex with Rictor in the absence of Raptor. We have begun to evaluate the effects of loss of Raptor on endothelial cell function and like we found for rapamycin treatment, there is a significant but incomplete inhibition of cellular proliferation, **Figure 7B**, suggesting at the level of S-phase entry, there is not a complete dependence on mTOR activity for cell cycle progression, at least in the presence of a strong agonist such as complete growth medium. We did find however that the loss of Raptor significantly inhibited the number, length and apparent branching, of vascular structures when endothelial cells were co-cultured with fibroblasts, as demonstrated in **Figure 8**. This experiment is consistent with growth inhibition, though more pronounced than the BrdU phenotype. We also used the drug-inducibility of this construct to probe the role of Raptor in the maintenance of vascular like morphology. Interestingly, when Raptor knockdown was induced after vascular morphogenesis was complete (14 days), we saw little or no effect of eliminating raptor expression, even when comparing identical fields. Thus, in resting or mature vessels mTORC1 seems to have little role in maintaining these quiescent

structures. We are currently making the cells with the dual knockdown of NF1 and Raptor to confirm the role of mTORC1 in driving the sheet like phenotype that is suggested by our previously published data with rapamycin, as well as the above data presented above with Rheb and PTEN.

Rictor: We saved development of this reagent for last, as it seems likely to integrate across many of the other pathways under investigation, and we wished to have better characterization of those first so we could optimize our use of the Rictor-knockdown probes. We have identified 6 putative sequences to knockdown Rictor and have obtained clones in the TRIPZ inducible lentiviral vector. We are getting ready to begin the screening of these constructs for the best knockdown and will use the best two to generate infected HUVECs and begin their characterization in the coming months.

Summary of Aim 1 progress: We have made great progress and are almost complete with our reagent generation and characterization of cellular signaling and cellular function. There are replicates of experiments to be completed, and quantification of some results as well as analysis of some missing signals such as phospho-AMPK still to be done, as does the finalization of Rictor knockdown. In the coming months we will finish these up and turn our attention to the double-knockdowns of NF1/Rictor and NF1/Raptor. In addition we will continue the investigation of the effects of Rapamycin, metformin, and lovastatin on the signaling and phenotypes affected by NF1.

Proposed alterations to experimental plan: We wish to seek approval to add the analysis of a TSC1/2 knockdown experiment to the experimental plan if time and resources allow. We would like to evaluate if this might be a more critical manipulation of the mTOR axis than expression of Rheb allows and to compare the results with Rheb. Similarly, we recently have found that the FDA approved drug, Zolendronic acid, may act as a potent inhibition of mTOR related signaling in endothelial cells at low doses. As this represents another potential mode of manipulating the vascular anomalies associated with NF1 we would like to include this in our analysis.

Aim 2. Determine if NF1-deficient human endothelial cells have altered responses to TGF-b.

In the statement of work we proposed to evaluate changes in EndMT, Smad activation and any changes in the receptor expression. As we realized the complexity of this task, we determined that the most efficient approach would be to try and determine if there were defects in TGF-b signaling (Smad activation/EndMT) prior to doing a wholesale catalog of all the components in the cell that may or may not be changing, as this would allow us to prioritize where and what to look for. In addition we realized that TGF-b was a family of molecules, not simply TGF-b1, possibly interacting with at least two different receptor systems, ALK5 and ALK1. Therefore as a first step, we sought to distinguish conditions where we could evaluate the signaling through each of these systems. We evaluated the effects on a number of TGFb superfamily ligands including TGFb1, BMP2, BMP4, BMP9, and TGFb2 with respect to dose and time suing Smad2/3 phosphorylation and Smad 1/5 phosphorylation as readouts for ALK5 and ALK1 respectively. As shown in **Figure 10**, and in **Figure 11**, when we treat cells with low doses of TGFb2 and BMP9 we can selectively activate ALK5 and ALK1 respectively. When we analyze this signaling in NF1-kd cells we find, that the ALK5 signaling seems to be diminished and the

ALK1 signaling seems to be enhanced suggesting a change in the balance between these two pathways. However these changes are subtle and we will continue to gather quantitative data. This hypothesis is consistent with the loss of Endoglin mRNA and with the enhancement of CD109 we reported with our preliminary data. Therefore, we reasoned that if we inhibited ALK5 using an inhibitor of that receptor kinase activity, perhaps we could reproduce the morphogenic phenotype seen with NF1. As shown in **Figure 11**, the compound SB431542 is a potent and selective inhibitor of ALK5 signaling, but has no effect on ALK1. Therefore we treated cells plated in co-culture with the ALK5 inhibitor or vehicle control. Interestingly, incubation of the cells with this inhibitor did result in an enhancement in the "island" or sheet like structures found in these co-cultures, as shown in **Figure 12**. Ultimately the reveal of the subtle differences may be most effective through the monitoring of the integrated response to that signaling such as gene expression and EndMT. We have begun to evaluate the effects of the NF1 loss on EndMT. We first needed to establish condition where we achieved EndMT as this is not a widely reported phenomenon and conditions seemed to vary. We failed to see any clearly and demonstrable EndMT morphologically or through markers genes such as smooth muscle cell in response to TGFb1 or TGFb2 (two previously reported inducers of EndMT), see for example **Figure 13** where induction by TGFb2 is very weak. However when we combined an inflammatory cytokine (in this case we chose IL1) we were able to see a marked induction of what appears to be a significant EndMT response, as defined by a decrease in endothelial markers such as CD31and eNOS and an enhancement o0f mesenchymal markers such as N-cadherin and alpha smooth muscle actin. As seen in **Figure 13**, there did not seem to be a significant blunting or enhancement of this response when NF1 was knocked down, however this response was analyzed at a very early and likely incomplete time point. We are repeating these studies at longer time points to try and provide more dramatic changes in the markers and more definitive answers to the effects of NF1. In our SOW we also proposed to investigate CD109, a potential receptor for TGFb and modulator of signaling, which we had shown was upregulated in NF1-kd cells. We originally envisioned doing this late in the project, we moved forward on this earlier as we thought it could help rule this molecule in (or out) as an important aspect of the phenotype realized in the absence of NF1, and potentially allow us to mechanistically work from both ends. To this end we engineered a human CD109-expressing doxycycline-inducible lentiviral vector which we used to transduce human endothelial cells. While we found good induction of the CD109 protein in our cells, we were surprised to see little effect on SMAD mediated signaling, at least over the time course we investigated it (at this point only 30 minutes), as demonstrated in **Figure 14**. We had predicted based on the literature that it might alter ALK5 signaling (Bizet et al., 2011). We also evaluated these cells, as well as control cells in our morphogenic assay. Interestingly, here the cells did reveal a defect identical to that seen with loss of NF1 (and other conditions with upregulation of mTORC1), in the manifestation of decreased numbers of branching tubes and the appearance of islands of monolayers cells (**Figure 15**). This defect could be at least partially ameliorated by increasing the availability of TGFb2, a ligand for ALK5. Similarly, the inhibition of ALK5 with an antagonist results in sheets that can be induced to form networks again by the presence of TGFb2, yet the combination of antagonist and CD109 expression seems to be largely resistant to added TGFb2, remaining largely in island-like sheets of cells.

Aim 2 Summary: We have identified an EndMT paradigm which we can utilize to reveal if NF1 loss affects the process. We have also found that we can selectively isolate selective SMAD

signaling pathways mediated by the two principle ALK receptors in HUVECs, ALK5 and ALK1. Our early data suggests that there may be an imbalance in the relative levels of SMAD signaling following the loss of NF1, such that ALK5/Smad2/3 signaling is diminished in favor of ALK1/Smad1/5 signaling. The inhibition of ALK5 using a chemical inhibitor as well as the expression of Cd109, seem to support this notion as a potential significant contributor to the morphogenic defects, though more quantitative and mechanistic data are needed.

Aim 3: ***Evaluate the consequences of bi-allelic loss of NF1in the vascular endothelium.***

The experiments for this Aim were slightly delayed relative to our statement of work, mostly due to the change in availability status for a critical strain of mice. While awaiting funding the NF1 deletion strain was cryopreserved thus there was a delay in obtaining this strain as it was recovered and the number of recovered pups was half that expected resulting in lower than expected numbers to initiate the breeding of the parental strains. Nonetheless, the breeding colonies have been generated, **Figure 16** shows the genotyping of one of our current breeders and demonstrates our ability to generate offspring of the appropriate genotype which is Cad5:ER-Cre/Rosa26-tdTomato/NF1$^{flox/-}$. We have an initial cohort of animals which will be ready for tamoxifen induction, to initiate recombination in the endothelium, in several weeks.

Key Research and Accomplishments:

- Generation of vectors for and validation of the knockdown of Raptor in an inducible fashion
- Generation of vectors for the knockdown of PTEN and validation
- Generation of a vector for inducible expression of Rheb
- Initial Characterization of signaling and functions affected following loss of PTEN
- Intitial Characterization of signaling and functions affected by the expression of Rheb
- Initial characterization of signaling and function in cells where Raptor is knocked down
- Establishment of a paradigm to induce EndMT in primary endothelial cells
- Preliminary evaluation of the effect of NF1 loss on EndMT
- Establishment of conditions for selective ALK receptor activation
- Preliminary assessment of changes in SMAD signaling following loss of NF1
- Generation of vectors for the inducible expression of CD109 and validation
- Preliminary assessment of signaling and functional changes following induction of CD109
- Formulation of a working hypothesis that decreases in ALK5 related signaling maybe a driver of the abnormal morphogenic phenotype seen following the loss of NF1.
- Generation of endothelial specific, inducible, loss of NF1 in a haplo-insufficient background with an integrated reported for the monitoring of Cre-recombinase activity and endothelial cell marking. These mice represent the first model of bi-allelic loss in the vascular endothelium of an adult organism under study.

Reportable Outcomes:

There were no reportable outcomes during this period. We are considering several options for reporting our data in the coming year as it continues to mature. These include the possibility of presenting at the North American Vascular Biology Meeting in the Fall or the CTF meeting next Spring.

Conclusion:

We feel we have made excellent progress in addressing the Aims of the first two year, as outlined in our SOW. We have adjusted the timing of some of the experiments, prioritizing experiments which we feel, in the evolution of the information, will provide us information on the most efficient path forward.

At this point our data strongly support a critical role of mTORC1 signaling in being a driver of the pro-proliferative events in endothelial cells, as well as the dysmorphic phenotype observed when NF1 expression is lost. Interestingly our data also suggests that two of the well-known signals downstream of Ras activation, ERK and PI3K, are not the drivers of the morphogenic phenotype, as Rheb expression can induce defects in morphogenesis, without activation of either signal. We are well-positioned to evaluate the effects of the pharmacologic interventions as well as assess the effects of some of these changes in the double knockdown cells in the coming year. We are hoping to extend those studies to include the evaluation of TSC1/2 knockdown to better assess the possible contributions of mTOR activation as Rheb was relatively weak. In addition we hope to include zolendronic acid , a bisphosphonate currently clinicall used, in our pharmacologic manipulations.

Our data, suggest an intriguing and novel model, in that the knockdown of NF1 results in a dampening of ALK5 signaling (or at least phenocopies it). This may be through any number of mechanisms including the upregulation of CD109, the down regulation of an obligatory co-receptor such as Endoglin,, the surface expression of a ALK5 itself, or direct modulation of a critical signal transduction pathway. Determining which of these mechanistic possibilities is at play and how they may be regulated by mTOR, will be the focus of the work in front of us.

Lastly we have generated mice of a genotype that will allow us to precisely mimic the conditions by which a somatic mutation event in the endothelial cell(s) of a child or adult might lead to the development of a vascular anomaly. Our first cohort is ready for injection in a few weeks and we will be ready to begin the histological analysis later this year.

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Supporting Material: None

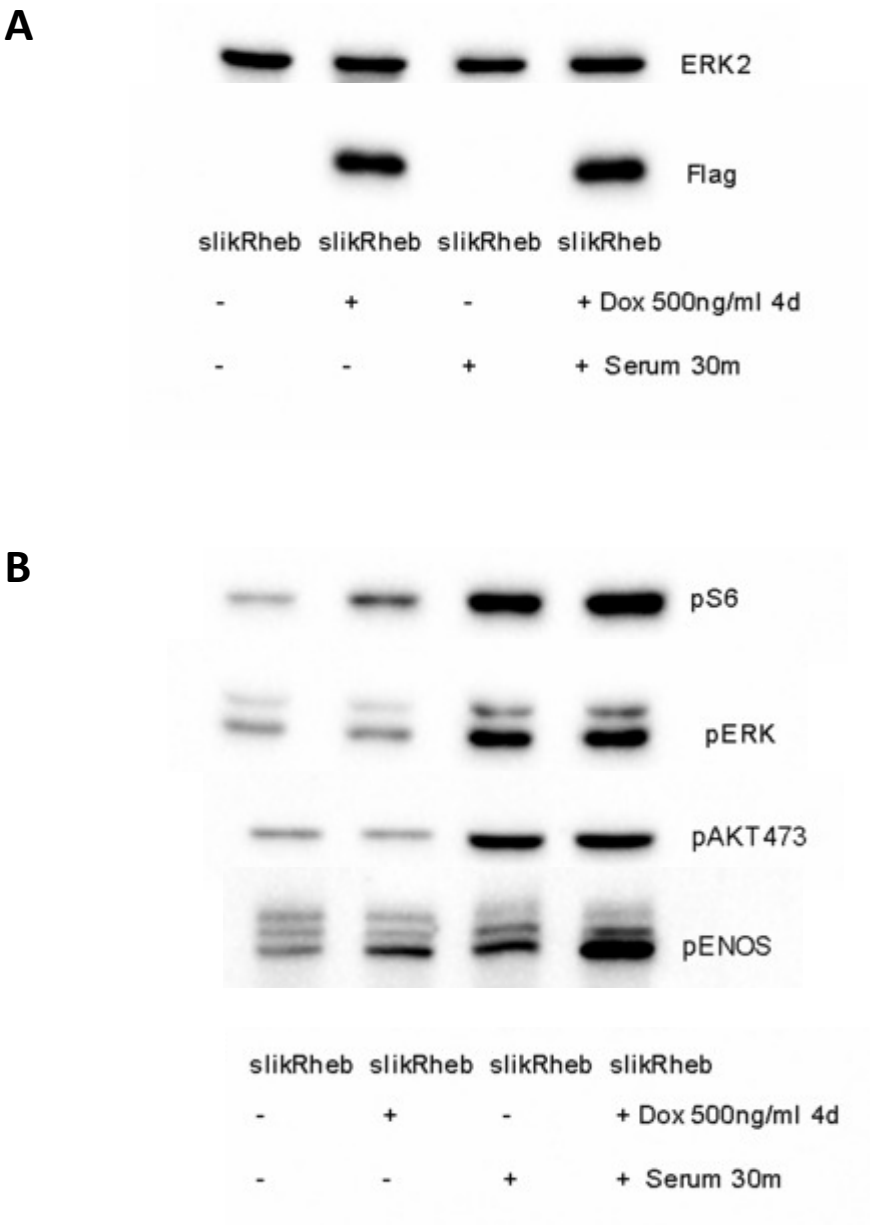


Figure 1 . Rheb induces modest activation of mTORC1. Low passage human umbilical endothelial cells were infected with a lentivirus coding for an epitope tagged (FLAG) Rheb protein expressed under doxycycline regulated conditions. Cells were induced to express Rheb for 4 days. On day three serum was removed and replaced with basal media for 16 hours prior to either continued quiescence or stimulation with serum in cells as indicated. Doxycycline regulated expression is confirmed in (A), using ERK expression to control for equivalent protein content in the lysates. In panel B these same lysates were probed with several potential signaling intermediates implicated as potentially important in vascular biology and morphogenesis and activated by the loss of NF1.

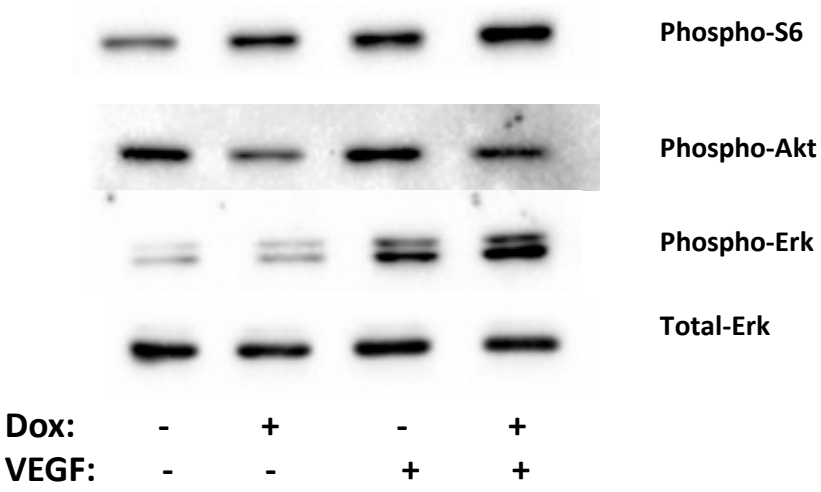
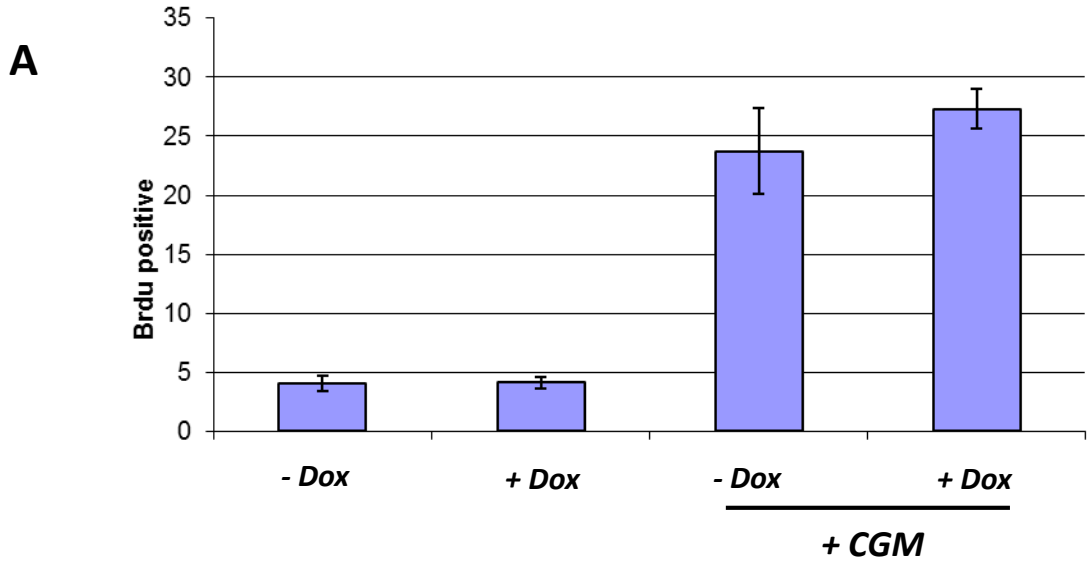


Figure 2. ***Rheb slightly augments VEGF mediated mTOR activation*** Cells infected with FLAG-Rheb were induced to express as indicated, and several signals were measured using western blotting. ERK was used as a loading control. This is representative of an experiment done twice.



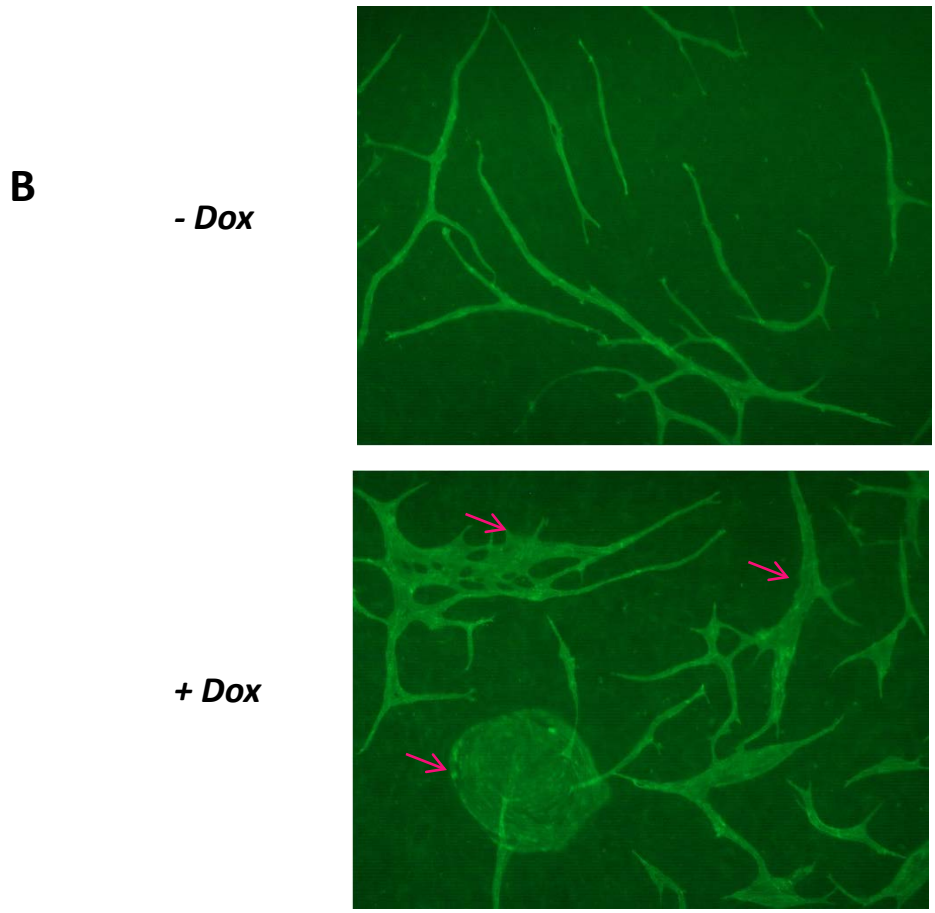
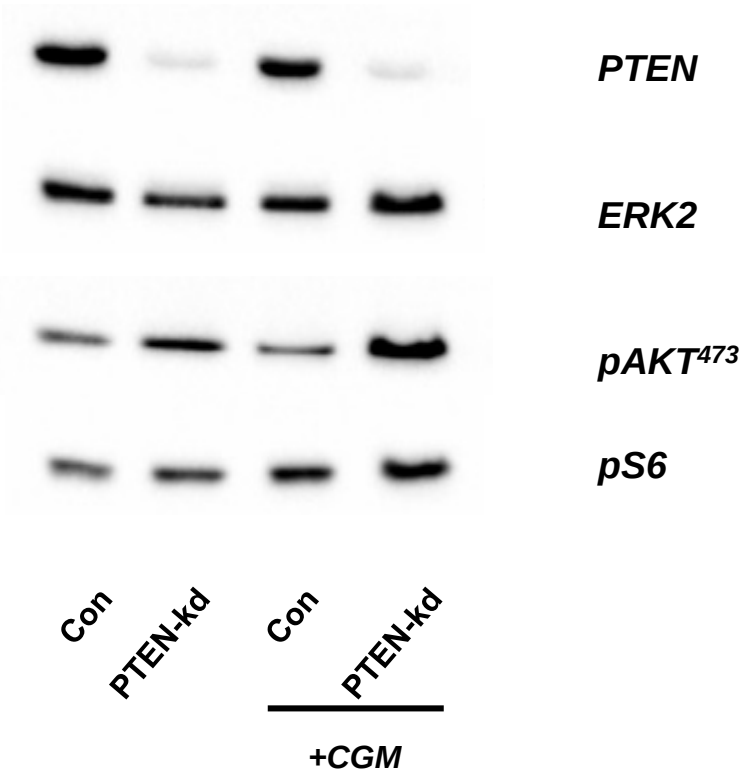


Figure 3. ***Activation of Rheb has modest effects on endothelial cell phenotype.*** HUVECs infected with lentivirus engineered to express Rheb in the presence of doxycycline were analyzed for their proliferative response **(A)** under basal conditions and in response to complete growth media (CGM). Data represent the average of three determinations +/- SD. In **(B)** the ability to form tubular networks in co-culture representing a vascular morphogenic response is analyzed. Examples of morphological defects are highlighted by arrows. Similar morphological phenotypes were seen in two additional experiments.

A



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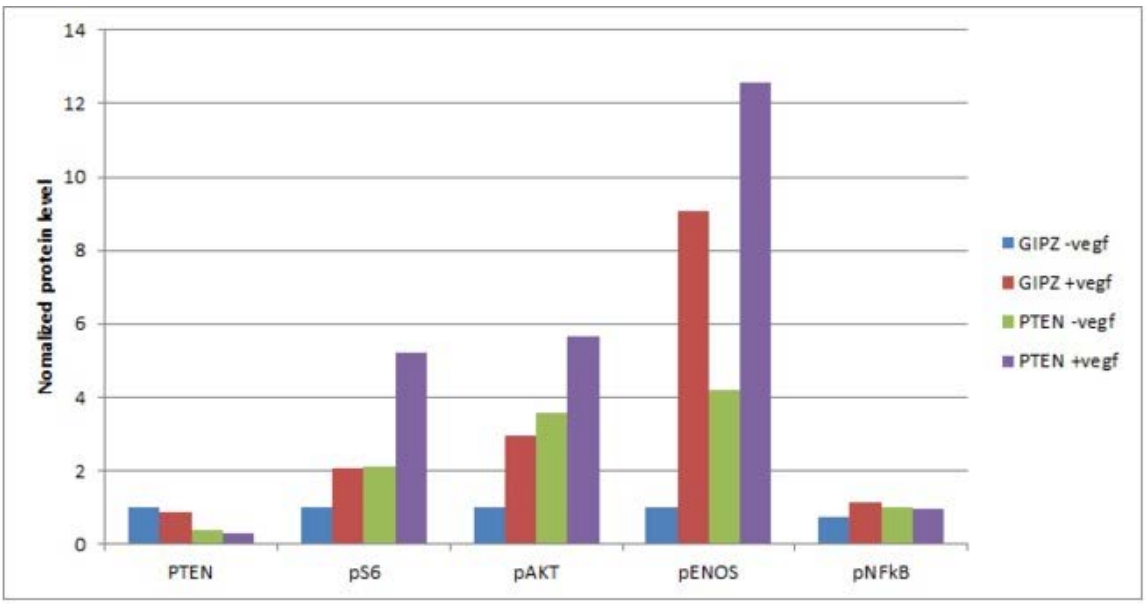
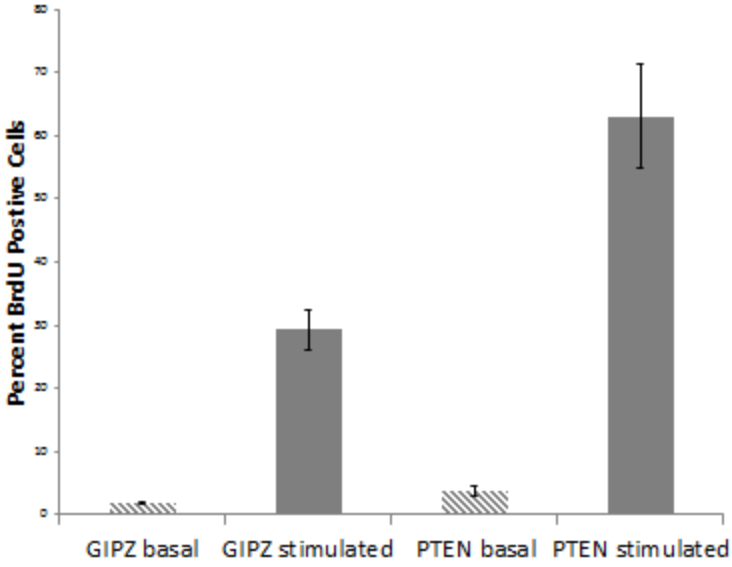


Figure 4. ***Analysis of Endothelial Cell Signaling following Lentiviral knockdown of PTEN***. **(A)** Cells infected with a lentivirus to knockdown PTEN expression were analyzed to verify knockdown and for the levels of several relevant cellular signals (A) by Western Blotting, following complete growth media. In addition **(B)** we analyzed these cells under basal and VEGF-stimulated conditions for activation of a number of different signals using western blotting. Lanes were quantified by chemiluminescence detection and normalized to expression of a housekeeping protein as a loading control.

A



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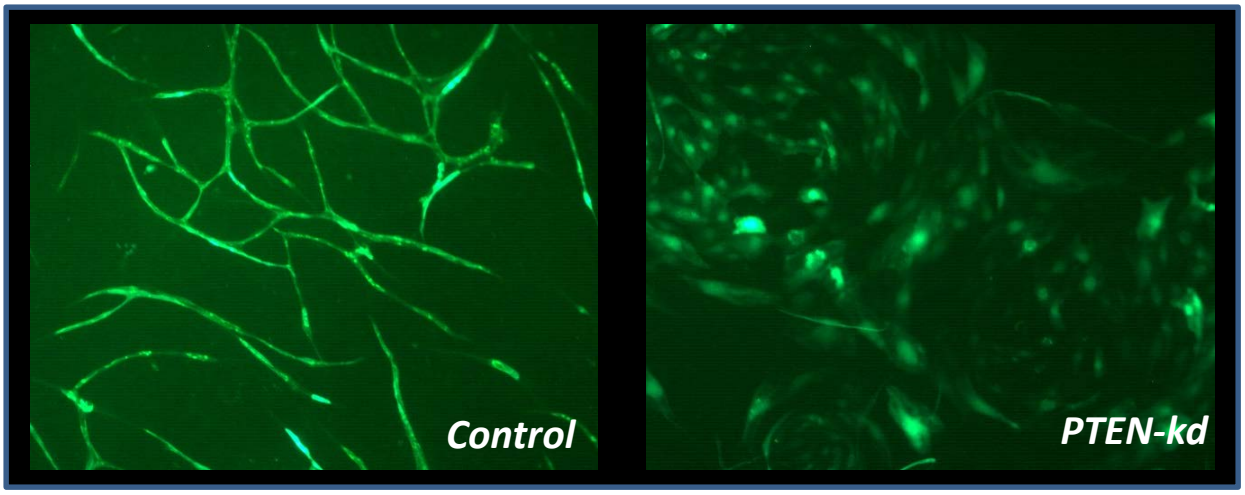


Figure 5 ***Loss of PTEN is sufficient to alter endothelial cell function and partially mimic the NF1 phenotype***. HUVECs infected with lenviruses coding for control mir-shRNA or one directed against PTEN were analyzed for entry into S phase as a marker of proliferation using BrdU incorporation (A) or for the formation of vascular-like structures when co-cultured with human fibroblasts

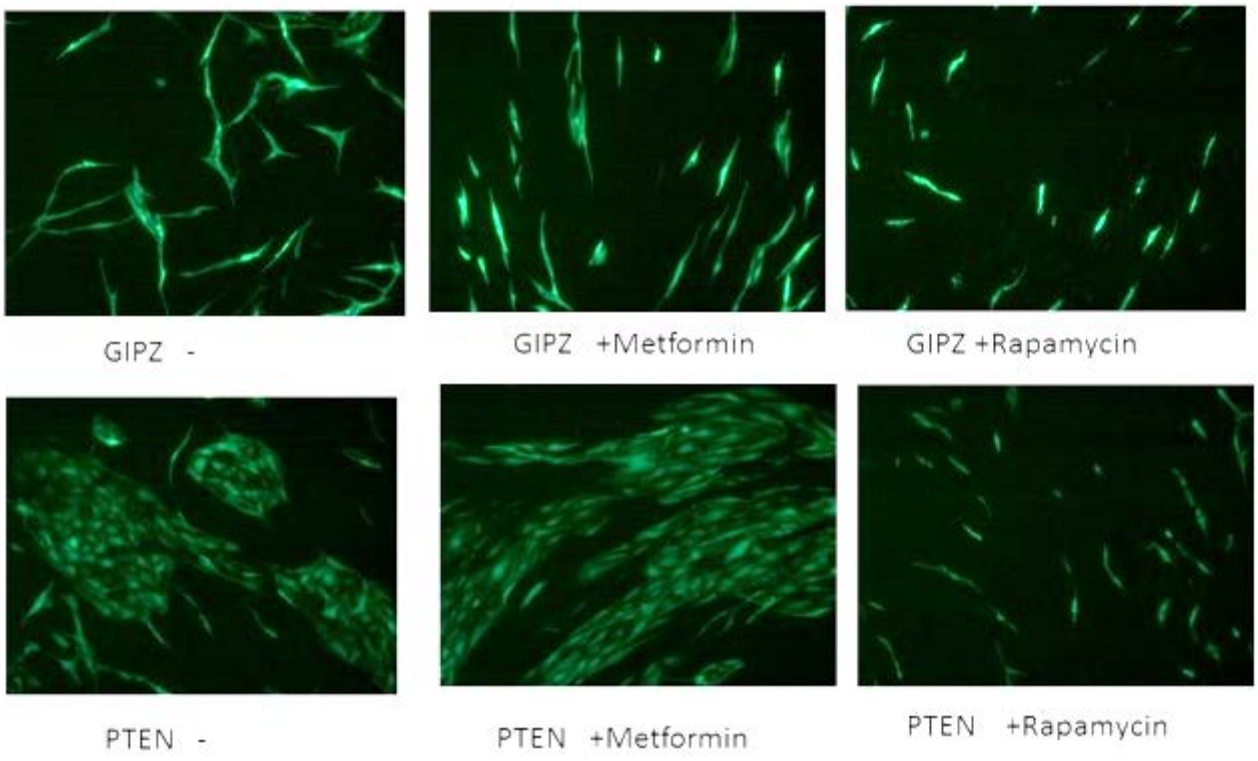


Figure 6. ***Normalization of defective morphogenesis following inhibition of mTORC1.*** GIPZ-control and PTEN-kd vectors were used to infect primary HUVECs. These cells were co-cultured with primary human fibroblasts in the presence of 1 mM metformin and 1 ng/ml Rpamycin. The co-expressed GFP was used to follow morphogenesis. These data, showing morphogenesis at 7 days.

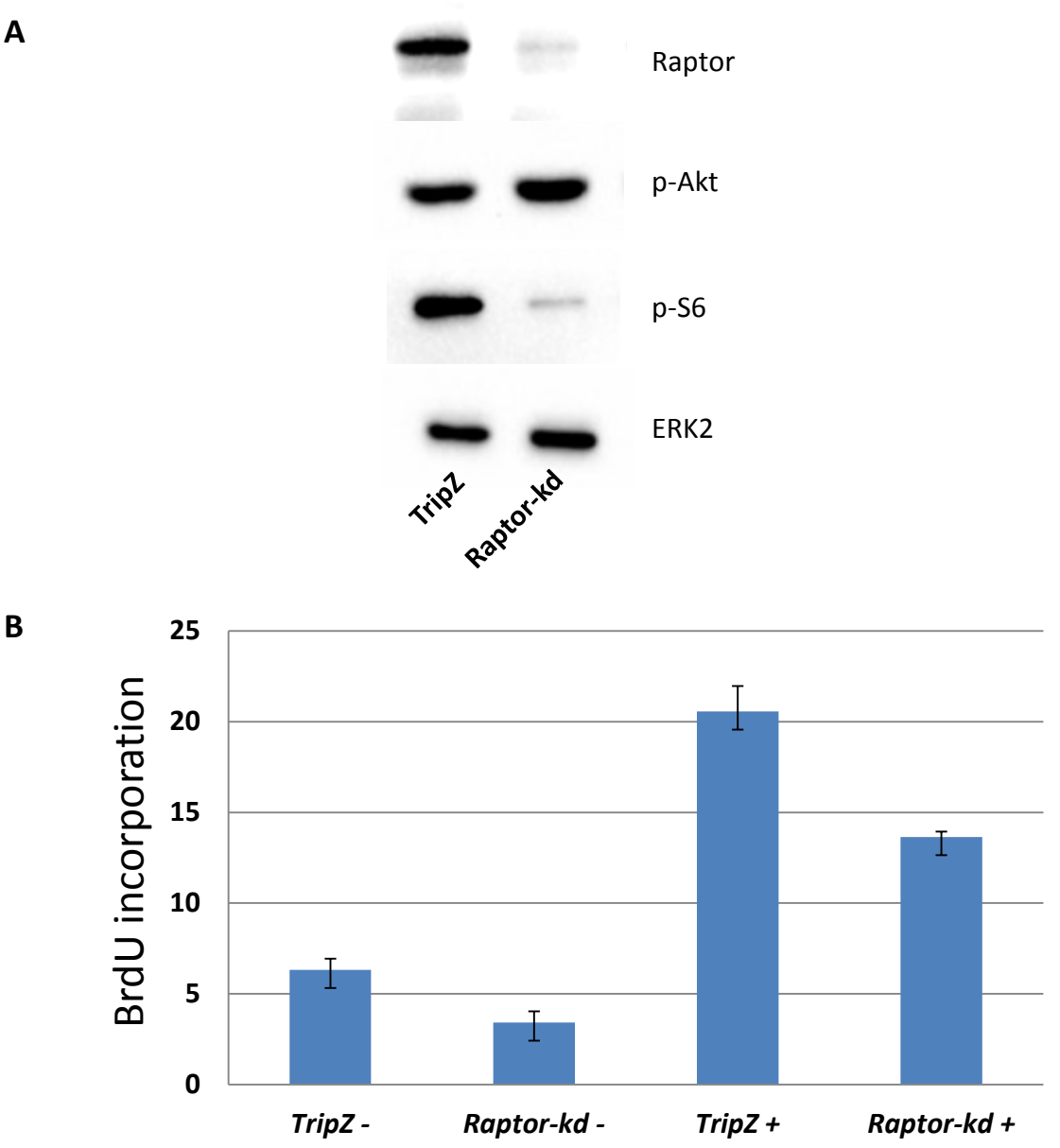
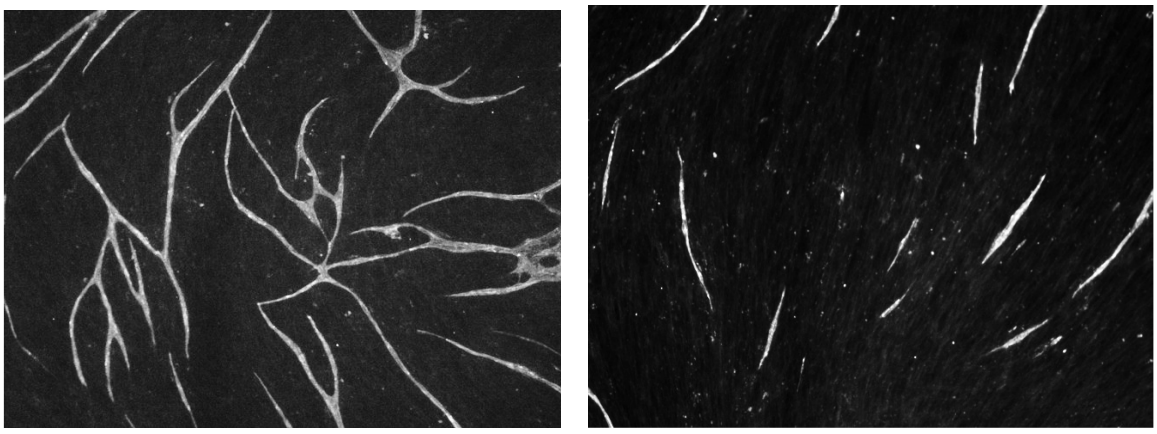
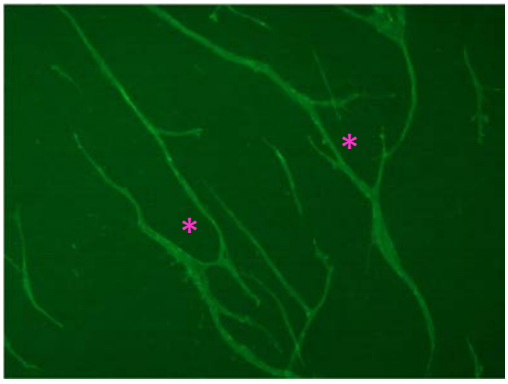


Figure 7. ***Effects of Raptor knockdown on cell signaling and proliferation.*** HUVECs were infected with lentiviruses coding for a control TRIPZ vector or a dox-inducible Raptor knockdown mir based shRNA. Infected cells grown in the presence of serum were analyzed for effects for efficiency of knockdown and cell signaling as indicated by labels on the Western blot **(A)**. In lower panel **(B)** these cells were stimulated with CGM to determine the requirement for Raptor for normal cell proliferation.

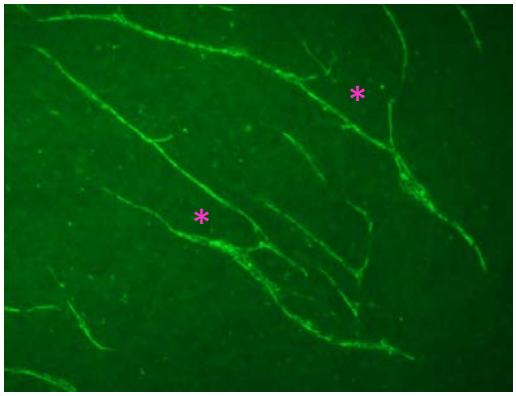


TRIPZ-Control **Raptor-kd**

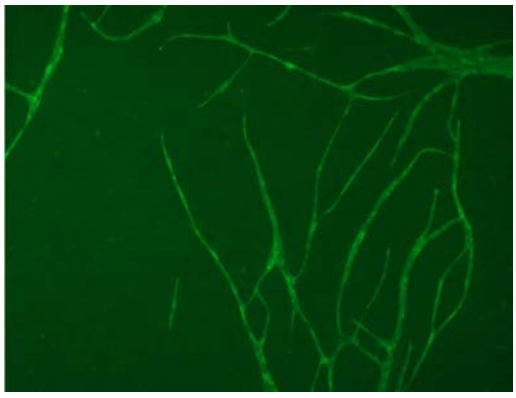
Figure 8*. Raptor knockdown results in diminished levels of vascular structures.* Primary endothelial cells infected with lentiviruses coding for control non-silencing shRNA or Raptor shRNA were induced with doxycycline. These cells co-express RFP upon induction which was used to track the formation of the vascular like networks formed upon co-culture with human fibroblasts. These images were captured 14 days after plating.



Raptor-kd
14 days – uninduced



Raptor-kd
14 days – uninduced
14 days – Induced



Control
14 days – uninduced
14 days – Induced

Figure 9. ***mTOR inhibition does not affect morphogenesis established vascular structures.*** HUVECs expressing doxycycline inducible shRNA targeting Raptor we co-cultured with human fibroblasts for 14 days in the absence of doxycycline to allow normal vascular morphogenesis. At day 14 they were stained live with UEA-1 lectin (FITC-labled) and photographed. Cells were then incubated with doxycycline to induce Raptor shRNA for 14 additional days. Cells were again stained live with UEA-lectin and similar fields were photographed. Asterisks mark prominent features for orientation. A culture of control cells was treated with a similar protocol for comparison.

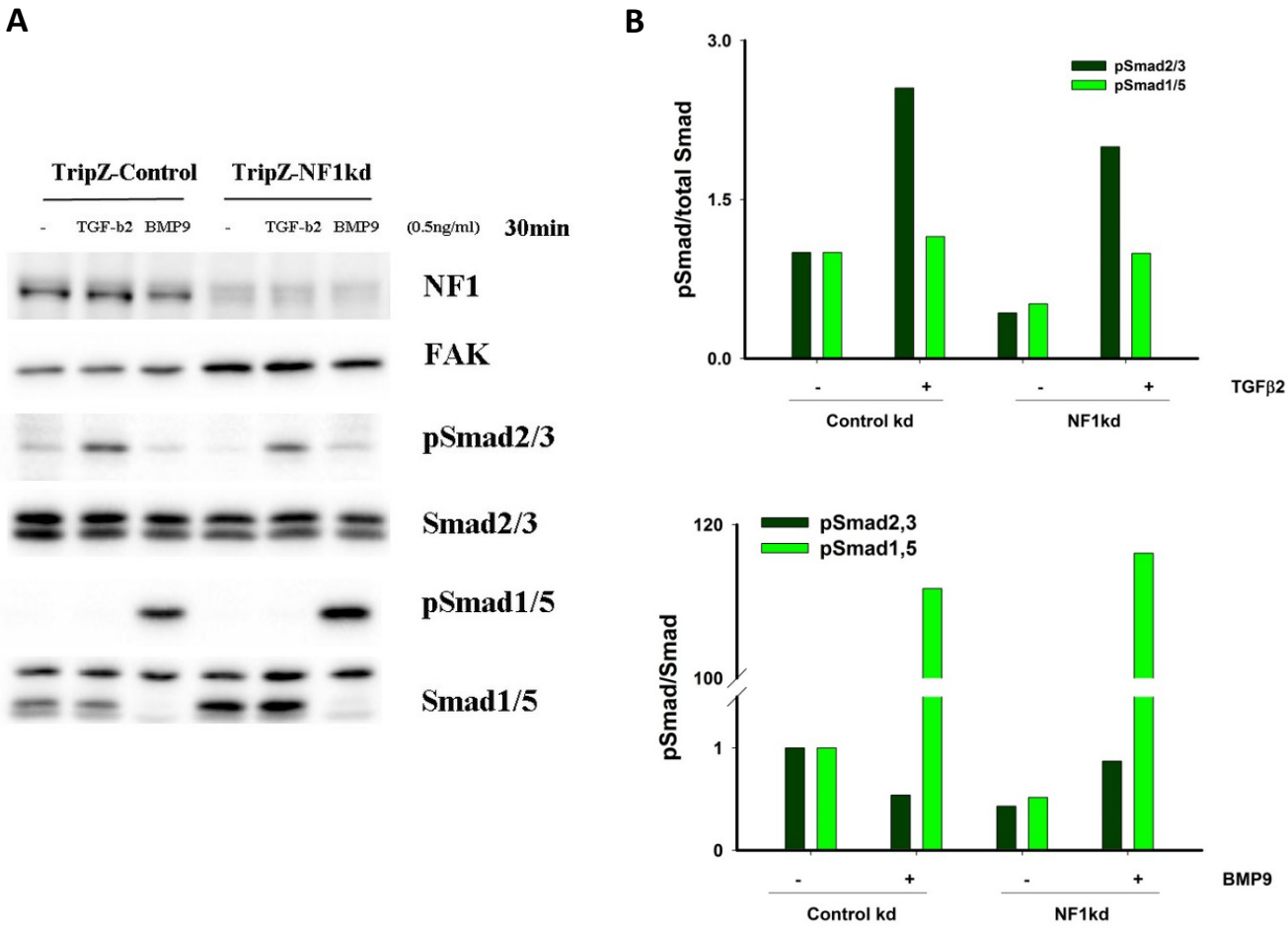


Figure 10. ***Modulation of the balance of TGF family signaling by loss of NF1***. **(A)** We used TGF-b2 and BMP9 at low doses and were able to demonstrate relatively selective signaling of Alk5 (SMAD 2/3) by TGFB2 and ALK1 (SMAD1/5) by BMP-9 at 30 minutes. Control HUVECs or those with knocked down NF1, were stimulated with BMP9 and TGF-b2 as indicated. **(B)** Results were quantified and normalized to total SMAD expression. These are preliminary results.

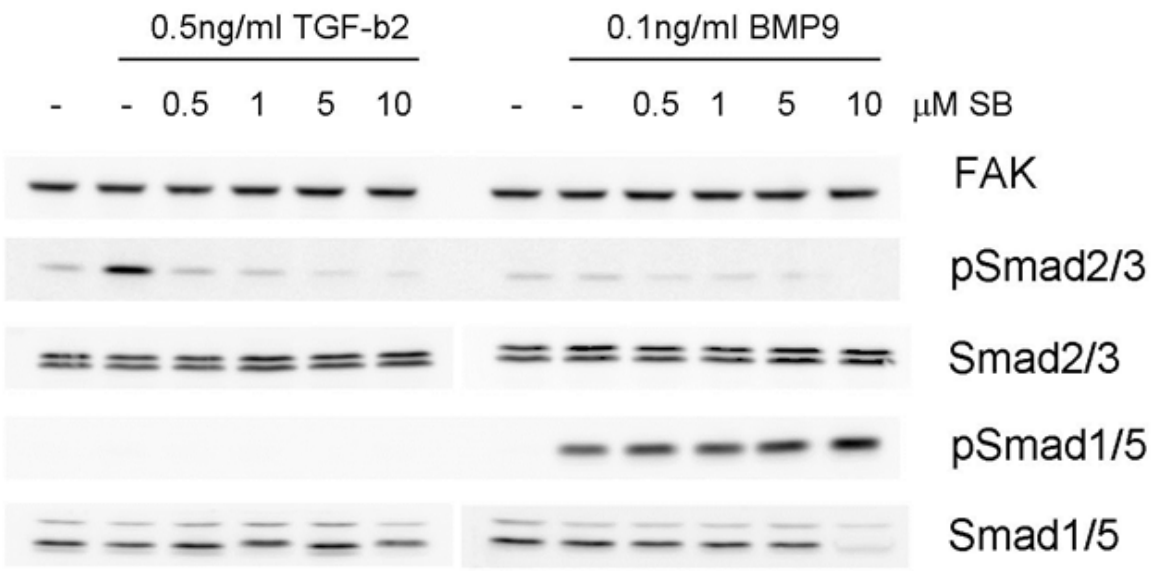
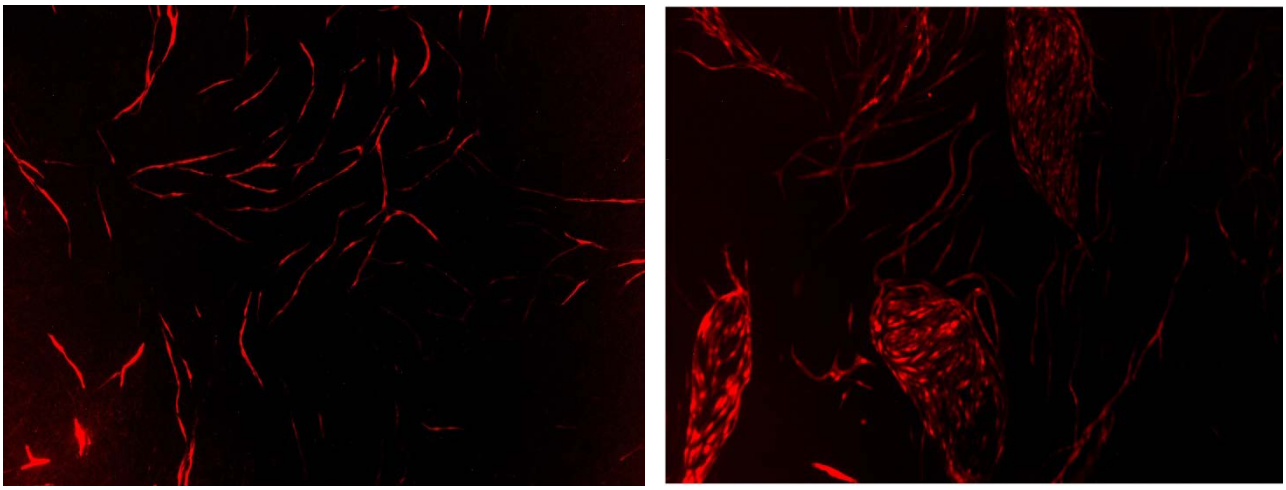


Figure 11. ***SB431542 is an effective and specific inhibitior of ALK5 signaling in human endothelial cells*****.** Cell were pretreated with indicated concentrations of SB431542 for 30 minutes prior to stimulation with either TGF-b2 or BMP9 to activate ALK5 or ALK1 respectively. Results were analyzed by western blotting.



Control ***SB431542 – 1 μM***

Figure 12. Inhibition of ALK5 disrupts vascular morphogenesis and mimics NF1 phenotype. RFP expressing primary endothelial cells were co-cultured with primary human fibroblasts for 14 days, during which time endothelial cells typically form anatamosing tubular networks that resemble vascular structures in vivo and which contain patent lumens. In this experiment cells were treated with either vehicle (Control) or with the ALK5 inhibitor, SB431542 at 1 μM

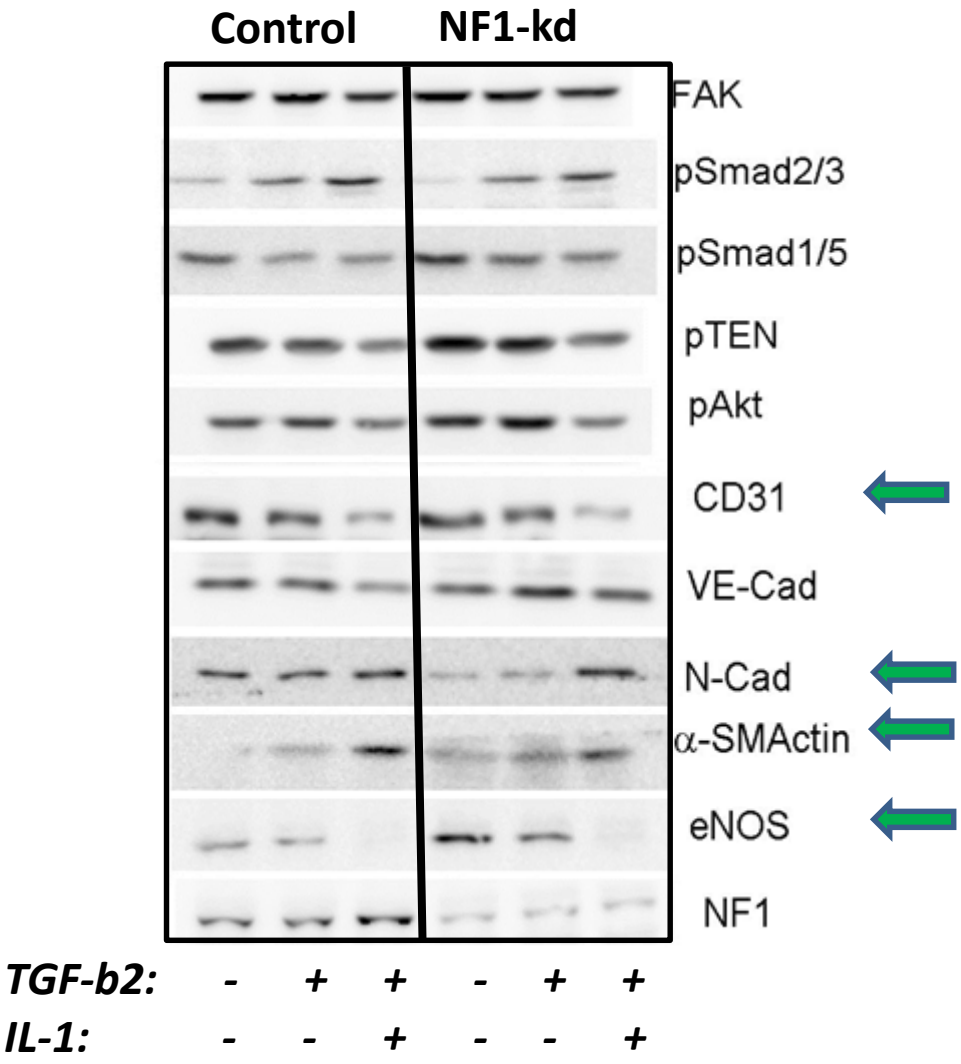


Figure 13. ***Induction of EndMT phenotype with little effect of NF1-kd***. Cells were treated with TGF-b2 or a combination of TGF-b2 and IL1, in an effort to induce a phenotype shift in the cells termed EndMT. This manifests itself as the gain of a number of stromal mesenchymal markers such as N-Cadherin and smooth muscle cell actin and the loss of endothelial specific markers such as CD31, VE-cadherin, and eNOS. Green arrows indicate blots with a clear EndMT shift in expression of the marker. These westerns were performed at a 3-day time point after treatment.

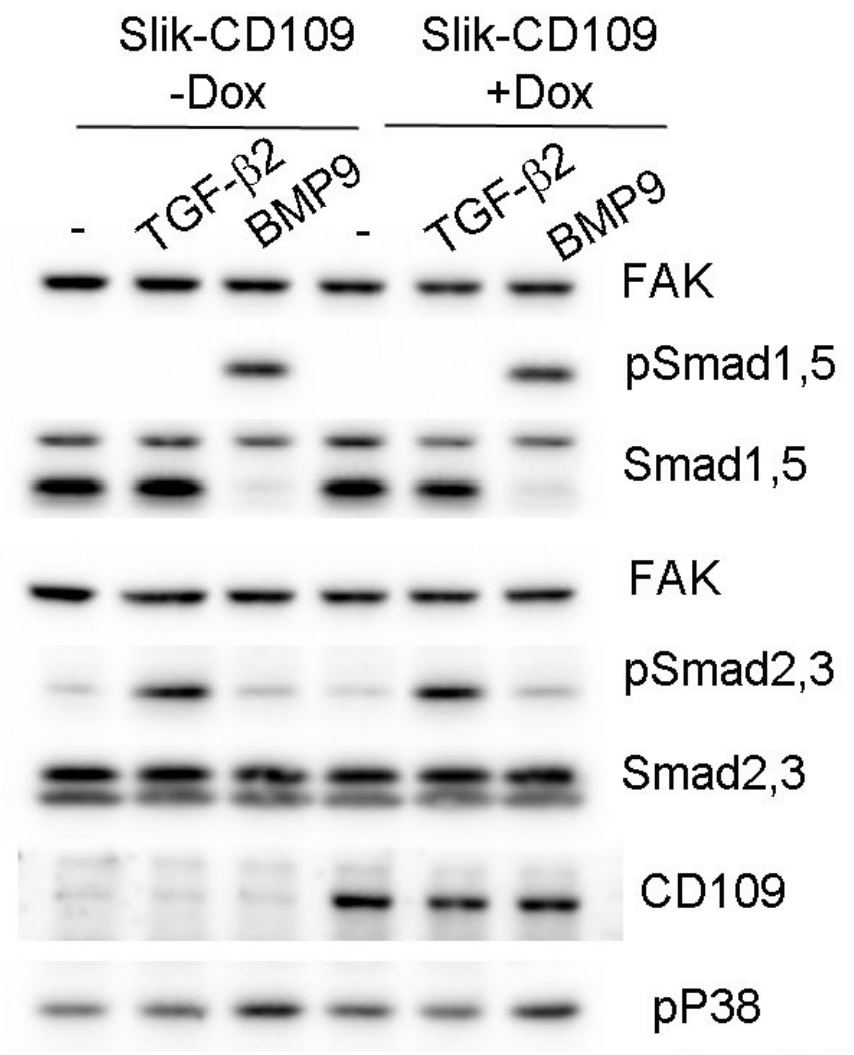


Figure 14. ***Characterization of effect of CD109 expression of SMAD signaling.*** HUVECs engineered to inducibly express cd109, were evaluated for regulation of cd109 expression following serum starvation and selective ALK stimulation for 30 minutes with either TGFb2 or BMP9. protein phosphorylation and expression was evaluated by western blotting for the proteins indicated on the right margin.

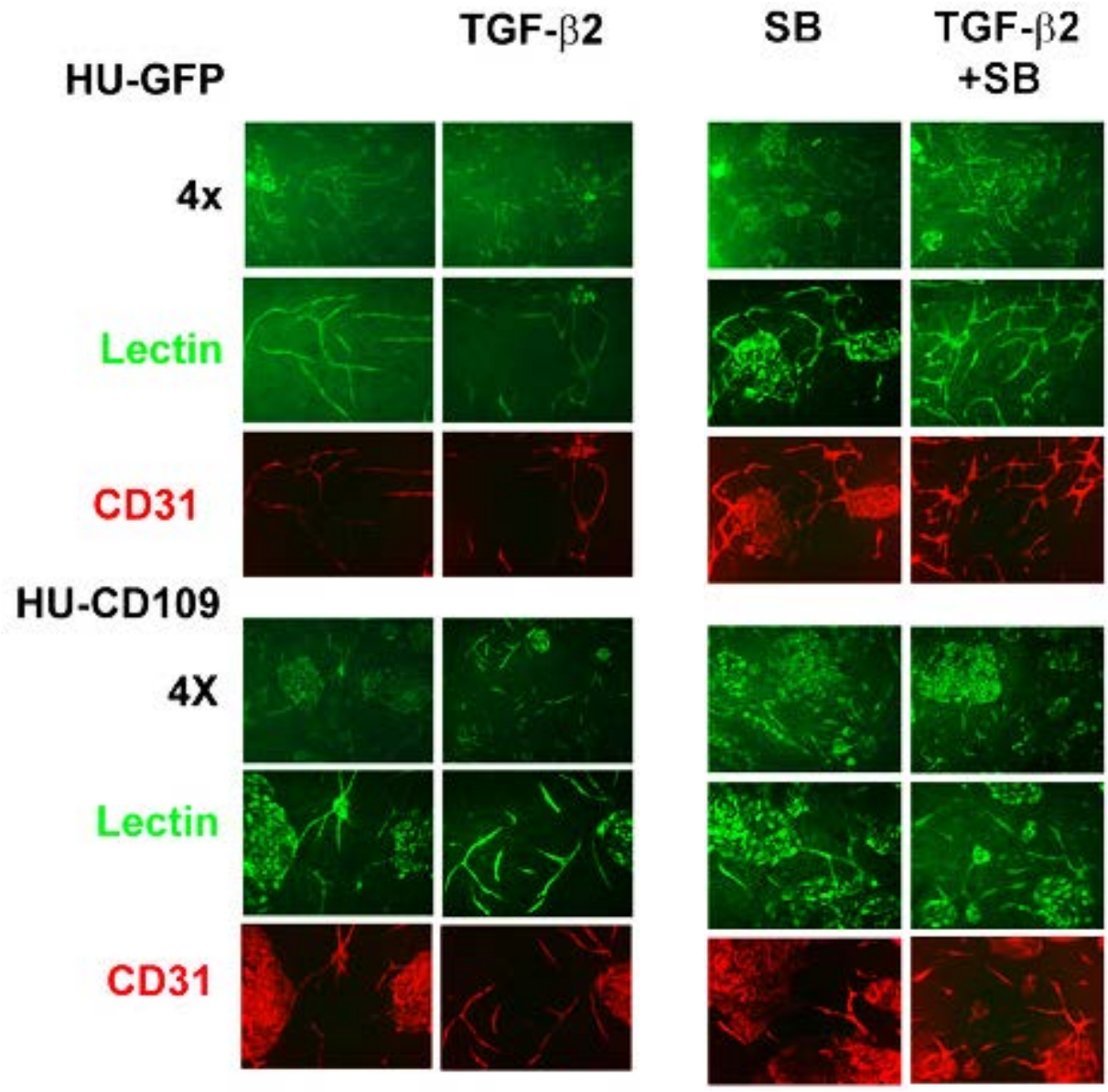


Figure 15. ***CD109 expression is sufficient to induce morphogenic defect, potentially through ALK5 suppression***. Control cells or cells induced to express CD109 were co-cultured with human fibroblasts in the presence of agonsits (TGF-b2) or antagonists (SB431542) of ALK5. Cells were stained live with lectin and then the same fields were fixed and stained with CD31 for confirmation. Images were acquired at 100X, as well as at 40X to visualize larger fields.

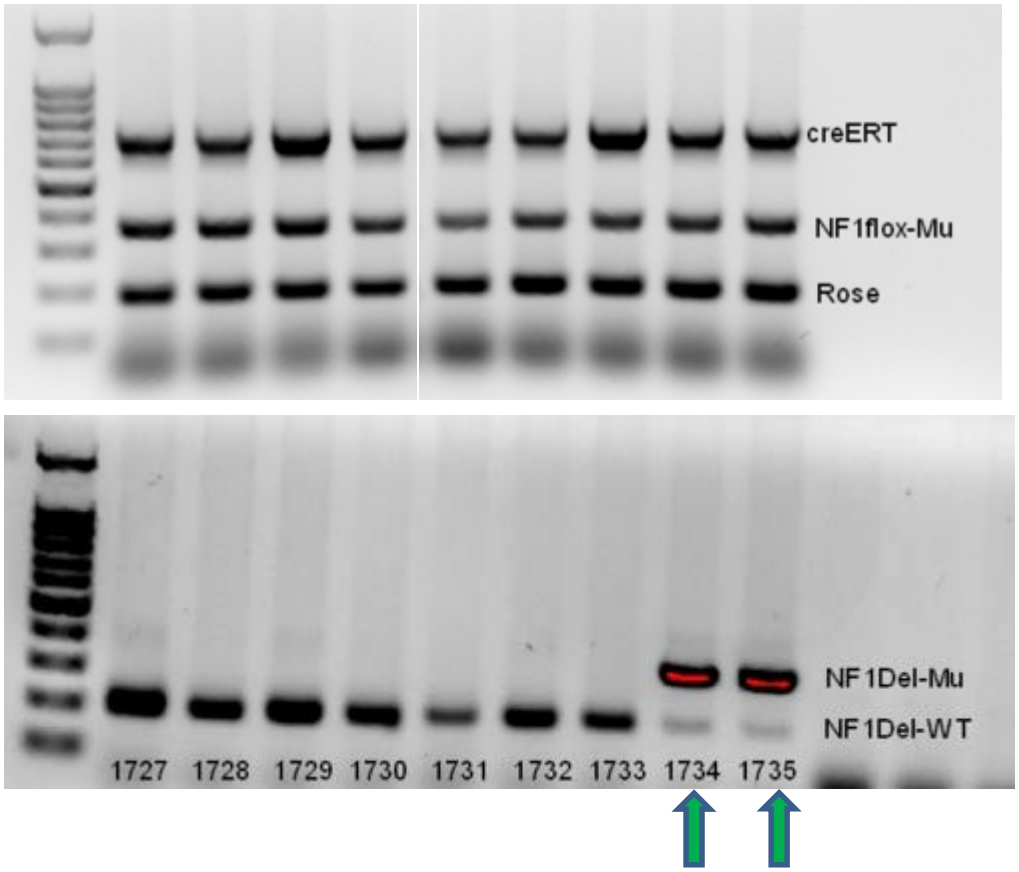


Figure XX ***Generation of Mice with Inducible, endothelial specific loss of NF1 on a heterozygotic background***. A litter of Mice was genotyped by Tail PCR for the presence of the Cad5:ERT allele, the Rosa26 LSL-tomato reporter, a floxed NF1 allele and the presence of a NF1 mutant (deletion) allele. Green arrows indicate mouse pups positive for all 4 selected alleles.